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APPLICATION FOR LETTERS PATENT

for

# SELF-CONTAINING LACTOCOCCUS STRAIN

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TITLE OF THE INVENTION

# SELF-CONTAINING LACTOCOCCUS STRAIN

## CROSS-REFERENCE TO RELATED APPLICATION

**[0001]** This application is a continuation of PCT International Patent Application No. PCT/EP02/04942, filed on May 3, 2002, designating the United States of America, and published, in English, as PCT International Publication No. WO 02/090551 A2 on November 14, 2002, the contents of the entirety of which are incorporated herein by this reference.

## TECHNICAL FIELD

**[0002]** The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where the medium compounds are present, but cannot survive outside the host organism in an absence of the medium compounds. Moreover, the *Lactococcus* can be transformed with prophylactic and/or therapeutic molecules and can, as such, be used to treat diseases such as inflammatory bowel diseases.

## BACKGROUND

**[0003]** Lactic acid bacteria have long been used in a wide variety of industrial fermentation processes. They have generally-regarded-as-safe ("GRAS") status, making them potentially useful organisms for the production of commercially important proteins. Indeed, several heterologous proteins, such as Interleukin-2, have been successfully produced in *Lactococcus* spp (Steidler *et al.*, 1995). It is, however, undesirable that such genetically modified microorganisms survive and spread into the environment.

**[0004]** To avoid unintentional release of genetically modified microorganisms, special guidelines for safe handling and technical requirements for physical containment are used. Although this may be useful in industrial fermentations, the physical containment is generally

considered as insufficient, and additional biological containment measures are taken to reduce the possibility of survival of the genetically modified microorganism in the environment.

**[0005]** Biological containment is extremely important in cases where physical containment is difficult or even inapplicable. This is, amongst others, the case in applications where genetically modified microorganisms are used as live vaccines or as a vehicle for delivery of therapeutic compounds. Such applications have been described, for example, in PCT International Publication Number WO 97/14806, which discloses the delivery of biologically active peptides, such as cytokines, to a subject by recombinant noninvasive or nonpathogenic bacteria. Further, PCT International Publication Number WO 96/11277 describes the delivery of therapeutic compounds to an animal or human by administering a recombinant bacterium encoding a therapeutic protein. Steidler *et al.* (2000) describe the treatment of colitis by administration of a recombinant *Lactococcus lactis* secreting Interleukin-10. Such a delivery may indeed be extremely useful to treat a disease in an affected human or animal, but the recombinant bacterium may act as a harmful and pathogenic microorganism when it enters a nonaffected subject, and an efficient biological containment that avoids such unintentional spreading of the microorganism is needed.

**[0006]** Biological containment systems for host organisms may be passive and based on a strict requirement of the host for a specific growth factor or a nutrient that is not present or is present in low concentrations in the outside environment. Alternatively, it may be active and, based on so-called suicidal genetic elements in the host, wherein the host is killed in the outside environment by a cell-killing function, encoded by a gene that is under the control of a promoter only being expressed under specific environmental conditions.

**[0007]** Passive biological containment systems are well known in microorganisms such as *Escherichia coli* or *Saccharomyces cerevisiae.* Such *E. coli* strains are disclosed, for example, in U.S. Patent 4,190,495. Also, PCT International Publication Number WO 95/10621 discloses lactic acid bacterial suppressor mutants and their use as means of containment in lactic acid bacteria, but in that case, the containment is on the plasmid level, rather than on the level of the host strain and it stabilizes the plasmid in the host strain, but does not provide containment for the genetically modified host strain itself.

**[0008]** Active suicidal systems have been described by several authors. Such systems consist of two elements: a lethal gene and a control sequence that switches on the expression of

the lethal gene under nonpermissive conditions. For example, PCT International Publication Number WO 95/10614 discloses the use of a cytoplasmatically active truncated and/or mutated *Staphylococcus aureus* nuclease as a lethal gene. PCT International Publication Number WO 96/40947 discloses a recombinant bacterial system with environmentally limited viability, based on the expression of either an essential gene, expressed when the cell is in the permissive environment and not expressed or temporarily expressed when the cell is in the nonpermissive environment, and/or a lethal gene, wherein expression of the gene is lethal to the cell and the lethal gene is expressed when the cell is in the nonpermissive environment but not when the cell is in the permissive environment. PCT International Publication Number WO 99/58652 describes a biological containment system based on the relE cytotoxin. However, most systems have been elaborated for *Escherichia coli* (Tedkin *et al.*, 1995; Knudsen *et al.*, 1995; Schweder *et al.*, 1995) or for *Pseudomonas* (Kaplan *et al.*, 1999; Molino *et al.*, 1998). Although several of the containment systems theoretically can by applied to lactic acid bacteria, no specific biological containment system for *Lactococcus* has been described that allows the usage of a self-containing and transformed *Lactococcus* to deliver prophylactic and/or therapeutic molecules in order to prevent and/or treat diseases.

## DISCLOSURE OF THE INVENTION

**[0009]** The invention includes a suitable biological containment system for *Lactococcus*. A first aspect of the invention is an isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene.

**[0010]** Another aspect of the invention is the use of a strain according to the invention as a host strain for transformation, wherein the transforming plasmid does not comprise an intact thymidylate synthase gene.

**[0011]** Still another aspect of the invention is a transformed strain of *Lactococcus* sp. according to the invention, comprising a plasmid that does not comprise an intact thymidylate synthase gene. Another aspect of the invention relates to a transformed strain of *Lactococcus* sp. comprising a gene or expression unit encoding a prophylactic and/or therapeutic molecule such as Interleukin-10. Consequently, the present invention also relates to the usage of a transformed strain of *Lactococcus* sp. to deliver prophylactic and/or therapeutic molecules and, as such, to treat diseases. Methods to deliver the molecules and methods to treat diseases such as

inflammatory bowel diseases are explained in detail in PCT International Publication Numbers WO 97/14806 and WO 00/23471 to Steidler *et al.* and in Steidler *et al.* (Science 2000, 289:1352), the contents of all of which are incorporated herein by this reference.

**[0012]** Another aspect of the invention is a medical preparation comprising a transformed strain of *Lactococcus* sp., according to the invention.

**[0013]** The invention further demonstrates that the transformed strains surprisingly pass the gut at the same speed as the control strains, showing that their loss of viability indeed is not different from that of the control strains. However, once the strain is secreted in the environment, for example, in the feces, it is not able to survive any longer.

**[0014]** The transforming plasmid can be any plasmid, as long as it does not complement the *thyA* mutation. It may be a self-replicating plasmid that preferably carries one or more genes of interest and one or more resistance markers, or it may be an integrative plasmid. In the latter case, the integrative plasmid itself may be used to create the mutation by causing integration at the *thyA* site, whereby the *thyA* gene is inactivated.

## BRIEF DESCRIPTION OF THE FIGURES

**[0015]** FIG. 1: Map of the MG1363 *thyA* locus.

**[0016]** FIG. 2: Schematic representation of the different expression modules as present on pOThy plasmids ands genomic integrants of hIL-10. Black parts represent original *L. lactis* MG1363 genetic information; white parts represent recombinant genetic information.

**[0017]** FIGS. 3A and 3B: PCR identification of Thy11 (Thy11 1.1 and Thy11 7.1 represent individually obtained, identical clones). Standard PCR reactions were performed by using aliquots of saturated cultures of the indicated strains as a source of a DNA template. FIG. 3A shows an agarose gel of the products of the indicated PCR reactions. FIG. 3B shows the positions at which primers attach in the thyA (1), upstream (2) or downstream (3) PCR's. Oligonucleotide primers used:

(1): ATgACTTACgCAgATCAAgTTTTT (SEQ ID NO:8 of the accompanying SEQUENCE LISTING, which is incorporated herein by this reference and TTAAATTgCTAAATCAAATTTCAATTg (SEQ ID NO:9)

(2): TCTgATTgAgTACCTTgACC (SEQ ID NO:10) and gCAATCATAATTggTTTTATTg (SEQ ID NO:11)

(3): CTTACATgACTATgAAAATCCg (SEQ ID NO:12) and cTTTTTTATTATTAgggAAAgCA (SEQ ID NO:13).

**[0018]** FIGS. 4A and 4B: PCR identification of Thy11, Thy12, Thy15 and Thy16. Standard PCR reactions were performed by using three-day old colonies of the indicated strains as a source of DNA template. FIG. 4A shows the positions at which primers attach in the upstream (1), downstream (2) or thyA (3), PCRs. Oligonucleotide primers used:

(1): ATgACTTACgCAgATCAAgTTTTT (SEQ ID NO:8) and TTAAATTgCTAAATCAAATTTCAATTg (SEQ ID NO:9)

(2): TCTgATTgAgTACCTTgACC (SEQ ID NO:10) and gCAATCATAATTggTTTTATTg (SEQ ID NO:11)

(3): CTTACATgACTATgAAAATCCg (SEQ ID NO:12) and cTTTTTTATTATTAgggAAAgCA (SEQ ID NO:13).

FIG. 4B shows an agarose gel of the products of the indicated PCR reactions.

**[0019]** FIGS. 5A and 5B: Southern blot analysis of the indicated strains. Chromosomal DNA was extracted and digested with the indicated restriction enzymes. Following agarose gel electrophoresis, the DNA was transferred to a membrane and the chromosome structure around the thyA locus was revealed by use of DIG-labeled thyA or hIL-10 DNA fragments (FIGS. 5A). FIG. 5B shows a schematic overview of the predicted structure of the thyA locus in both MG1363 and Thy11.

**[0020]** FIG. 6A shows a schematic overview of part of the predicted structure of the *L. lactis* chromosome at the *thy*A locus in MG1363, Thy11, Thy12, Thy15 and Thy16. Numbers indicate base pairs. FIG. 6B illustrates a Southern blot analysis of the indicated strains. Chromosomal DNA was extracted and digested with *Nde*I and *Spe*I restriction enzymes. Following agarose gel electrophoresis, the DNA was transferred to a membrane and the chromosome structure around the thyA locus was revealed by use of DIG-labeled thyA or hIL-10 DNA fragments.

**[0021]** FIGS. 7A and 7B: Production of hIL-10. FIG. 7A shows a western blot revealed with anti-hIL-10 antiserum of culture supernatant and cell-associated proteins of the indicated strains. FIG. 7B shows quantification (by ELISA) of hIL-10 present in the culture supernatant.

**[0022]** FIGS. 8A and 8B: Production of hIL-10. FIG. 8A shows quantification (by ELISA) of hIL-10 present in the culture supernatant of the indicated strains. FIG. 8B shows a western blot revealed with anti-hIL-10 antiserum of culture supernatant proteins of the indicated strains.

**[0023]** FIG. 9: Production of hIL-10 by the *L. lactis* strains LL108 carrying pOThy11, pOThy12, or pOThy16. Quantification (by ELISA) of hIL-10 present in the culture supernatant of the indicated strains is shown. The N-terminal protein sequence of the recombinant hIL-10 was determined by Edman degradation and was shown to be identical to the structure as predicted for the mature, recombinant hIL-10. The protein showed full biological activity.

**[0024]** FIGS. 10A and 10B: Growth rate of the indicated strains in GM17 containing 100 μg/ml (T100), 50 μg/ml (T50), 25 μg/ml (T25), or no (T0) extra thymidine and possibly supplemented with 5 μg/ml of erythromycin (E). Saturated overnight cultures (prepared in T50) were diluted 1:100 in the indicated culture media. FIG. 10A shows the kinetics of absorbance accumulation. FIG. 10B shows the kinetics of the number of colony-forming units (cfu) per ml of culture.

**[0025]** FIG. 11: Growth rate of MG1363 and Thy12 in thymidine-free medium (TFM). TFM was prepared by growing *L. lactis* Thy12 bacteria in GM17, removing the bacteria by subsequent centrifugation and filtration on a 0.22 μm pore size filter, adjusting the pH to 7.0 and autoclaving. MG1363 and Thy12 bacteria were collected from an overnight culture in GM17 or GM17+50 μg/ml of thymidine, respectively, and washed in M9 buffer (6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 1 g/l $NH_4Cl$, 0.5 g/l NaCl in water). The suspensions of both were either diluted in TFM or TFM supplemented with 50 μg/ml of thymidine (T50). CFU counts were determined at different time points: t=0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 20 hours. This shows that Thy12 viability is severely impaired in the absence of thymidine.

**[0026]** FIG. 12: Intestinal passage and viability: *L. lactis* MG1363 was transformed with the plasmid pLET2N, which carries a chloramphenicol (Cm) resistance marker. *L. lactis* Thy12 was transformed with the plasmid pT1NX, which carries an erythromycin (Em) resistance marker. Of both strains, $10^9$ bacteria were resuspended in BM9 (6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 1 g/l $NH_4Cl$, 0.5 g/l NaCl in 25 mM $NaHCO_3$ + 25 mM $Na_2CO_3$), mixed and inoculated in three mice at t=0h. Feces were collected at the time intervals -1 to 0, 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 9, 9 to 10 and 10 to overnight. All samples were resuspended in

isotonic buffer and appropriate dilutions were plated on GM17 (M17 medium, Difco, St. Louis, MO, supplemented with 0.5% glucose) plates containing either Cm, Em or Em+ 50 µg/ml thymidine. Colony-forming units for the different plates are represented in the graph.

## DETAILED DESCRIPTION OF THE INVENTION

**[0027]** As previously identified, the invention includes a suitable biological containment system for *Lactococcus*. In one aspect, the invention is an isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene. Preferably, the defective thymidylate synthase gene is inactivated by gene disruption. Even more preferably, the *Lactococcus* sp. is *Lactococcus lactis*. A special embodiment is a *Lactococcus* sp. strain, preferably *Lactococcus lactis*, more preferably a *Lactococcus lactis* MG1363 derivative, wherein the thymidylate synthase gene has been disrupted and replaced by an Interleukin-10 expression unit. The Interleukin-10 expression unit is preferably, but not limited to, a human Interleukin-10 expression unit or gene encoding for human Interleukin-10.

**[0028]** The *Lactococcus lactis* subsp. *lactis* thymidylate synthase gene (*thyA*) has been cloned by Ross *et al.* (1990a). Its sequence is comprised in SEQ ID NO:3 and SEQ ID NO:5. European Patent Application Publication Number 0406003 discloses a vector devoid of antibiotic resistance and bearing a thymidylate synthase gene as a selection marker; the same vector has been described by Ross *et al.* (1990b). However, this vector could not be used in a *Lactococcus lactis* strain due to the lack of a suitable *thyA* mutant that has never been described. The present invention discloses how to construct such a mutant by gene disruption using homologous recombination in *Lactococcus*. In a preferred embodiment, the *thyA* gene is disrupted by a functional human Interleukin-10 expression cassette. However, it is clear that any construct can be used for gene disruption, as long as it results in an inactivation of the *thyA* gene or in an inactive thymidylate synthase. As a nonlimiting example, the homologous recombination may result in a deletion of the gene, in one or more amino acid substitutions that lead to an inactive form of the thymidylate synthase, or in a frame shift mutation resulting in a truncated form of the protein.

**[0029]** Such a *Lactococcus* sp. *thyA* mutant is very useful as a host strain for transformation in situations where more severe containment than purely physical containment is needed. Indeed, *thyA* mutants cannot survive in an environment without or with only a limited

concentration of thymidine and/or thymine. When such a strain is transformed with a plasmid that does not comprise an intact *thyA* gene and cannot complement the mutation, the transformed strain will become suicidal in a thymidine/thymine-poor environment. Such a strain can be used in a fermentor as an additional protection for the physical containment. Moreover, the present invention discloses that such a strain is especially useful in cases where the strain is used as a delivery vehicle in an animal body. Indeed, when such a transformed strain is given, for example, orally to an animal – including humans – it survives in the gut, provided that a sufficiently high concentration of thymidine/thymine is present, and produces homologous and/or heterologous proteins, such as human Interleukin-10, that may be beneficial for the animal.

**[0030]** The invention further demonstrates that the transformed strains surprisingly pass the gut at the same speed as the control strains, showing that their loss of viability indeed is not different from that of the control strains. However, once the strain is secreted in the environment, for example, in the feces, it is not able to survive any longer.

**[0031]** The transforming plasmid can be any plasmid, as long as it does not complement the *thyA* mutation. It may be a self-replicating plasmid that preferably carries one or more genes of interest and one or more resistance markers, or it may be an integrative plasmid. In the latter case, the integrative plasmid itself may be used to create the mutation by causing integration at the *thyA* site, whereby the *thyA* gene is inactivated. Preferably, the active *thyA* gene is replaced by double homologous recombination by a cassette comprising the gene or genes of interest, flanked by targeting sequences that target the insertion to the *thyA* target site. It is of extreme importance that these sequences are sufficiently long and sufficiently homologous to integrate the sequence into the target site. Preferably, the targeting sequences include at least 100 contiguous nucleotides of SEQ ID NO:1 at one side of the gene of interest and at least 100 contiguous nucleotides of SEQ ID NO:2 at the other side. More preferably, the targeting sequences consist of at least 500 contiguous nucleotides of SEQ ID NO:1 at one side of the gene of interest and at least 500 contiguous nucleotides of SEQ ID NO:2 at the other side. Most preferably, the targeting sequences consist of SEQ ID NO:1 at one side of the gene of interest and SEQ ID NO:2 at the other side, or the targeting sequences consist of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID NO:1 at one side of the gene of interest and at least 100 nucleotides that are at least 80% identical,

preferably 90% identical to a region of SEQ ID NO:2 at the other side of the gene of interest. Preferably, the targeting sequences consist of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID NO:1 at one side of the gene of interest and at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID NO:2 at the other side of the gene of interest. Most preferably, the targeting sequences consist of at least 1000 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID NO:1 at one side of the gene of interest and at least 1000 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID NO:2 at the other side of the gene of interest. The percentage identity is measured with BLAST, according to Altschul *et al.* (1997). A preferred example of a sequence homologous to SEQ ID NO:1 is given in SEQ ID NO:7. For the purpose of the invention, SEQ ID NO:1 and SEQ ID NO:7 are interchangeable.

**[0032]** Transformation methods of *Lactococcus* are known to the person skilled in the art and include, but are not limited to, protoplast transformation and electroporation.

**[0033]** A transformed *Lactococcus* sp. strain according to the invention is useful for the delivery of prophylactic and/or therapeutic molecules and can be used in a pharmaceutical composition. The delivery of such molecules has been disclosed, as a nonlimiting example, in PCT International Publication Numbers WO 97/14806 and WO 98/31786. Prophylactic and/or therapeutic molecules include, but are not limited to, polypeptides such as insulin, growth hormone, prolactin, calcitonin, group 1 cytokines, group 2 cytokines and group 3 cytokines and polysaccharides such as polysaccharide antigens from pathogenic bacteria. A preferred embodiment is the use of a *Lactococcus* sp. strain according to the invention to deliver human Interleukin-10. This strain can be used in the manufacture of a medicament to treat Crohn's disease as indicated herein.

**[0034]** The invention is further explained with the use of the following illustrative examples.

## Examples

**[0035]** From *L. lactis* MG1363 (Gasson, 1983) regions flanking the sequence according to Ross *et al.* (1990a) have been cloned.

**[0036]** The knowledge of these sequences is of critical importance for the genetic engineering of any *Lactococcus* strain in a way as described below, as the strategy will employ double homologous recombination in the areas of 1000 bp at the 5′ end (SEQ ID NO:1) and 1000 bp at the 3′ end (SEQ ID NO:2) of thyA, the "thyA target." These sequences are not available from any public source to date. These flanking DNA fragments have been cloned and their sequence has been identified. The sequence of the whole locus is shown in SEQ ID NO:3; a mutant version of this sequence is shown in SEQ ID NO:5. Both the 5′ and 3′ sequences are different from the sequence at GenBank AE006385 describing the *L. lactis* IL1403 sequence (Bolotin, in press) or at AF336368 describing the *L. lactis* subsp. *lactis* CHCC373 sequence. From the literature, it is apparent that homologous recombination by use of the published sequences adjacent to *thyA* (Ross *et al.,* 1990a) (86 bp at the 5′ end and 31 bp at the 3′ end) is virtually impossible due to the shortness of the sequences. Indeed, Biswas *et al.* (1993) describe a logarithmically decreasing correlation between the length of the homologous sequences and the frequency of integration. The sequences of *L. lactis* Thy 11, Thy 12, Thy 15 and Thy 16 at the thyA locus as determined in the present invention are given by SEQ ID NOS:19, 20, 21, 22 respectively.

**[0037]** The *thyA* replacement is performed by making suitable replacements in a plasmid-borne version of the *thyA* target, as described below. The carrier plasmid is a derivative of pORI19 (Law *et al.*, 1995), a replication-defective plasmid, which only transfers the erythromycin resistance to a given strain when a first homologous recombination, at either the 5′ 1000 bp or at the 3′ 1000 bp of the *thyA* target. A second homologous recombination at the 3′ 1000 bp or at the 5′ 1000 bp of the *thyA* target yields the desired strain.

**[0038]** The *thyA* gene is replaced by a synthetic gene encoding a protein which has the *L. lactis* Usp45 secretion leader (van Asseldonk *et al.*, 1990) fused to a protein of an identical amino acid sequence when: (a) the mature part of human-Interleukin 10 (hIL-10) or (b) the mature part of hIL-10 in which proline at position 2 has been replaced with alanine or (c) the mature part of hIL-10 in which the first two amino acids have been deleted; (a), (b) and (c) are called hIL-10 analogs, the fusion products are called Usp45-hIL-10.

**[0039]** The *thyA* gene is replaced by an expression unit comprising the lactococcal P1 promoter (Waterfield *et al.*, 1995), the *E. coli* bacteriophageT7 expression signals, putative RNA stabilizing sequence and modified gene10 ribosomal binding site (Wells and Schofield, 1996).

**[0040]** At the 5′ end, the insertion is performed in such way that the ATG of thyA is fused to the P1-T7Usp45-hIL-10 expression unit.

5′ agataggaaaatttc**atg**acttacgcagatcaagttttt...thyA wild-type (SEQ ID NO:27)

gattaagtcatcttacctctt...P1-T7-usp45-hIL10 (SEQ ID NO:14)

5′ agataggaaaatttc**atg**gattaagtcatcttacctctt...thyA⁻,P1-T7-usp45-hIL10 (SEQ ID NO:15)

**[0041]** Alternatively, at the 5′ end, the insertion is performed in such a way that the thyA ATG is not included:

5′ agataggaaaatttcacttacgcagatcaagttttt...thyA wild-type (SEQ ID NO:28)

gattaagtcatcttacctctt...P1-T7-usp45-hIL10 (SEQ ID NO:14)

5′ agataggaaaatttcgattaagtcatcttacctctt...thyA⁻,P1-T7-usp45-hIL10 (SEQ ID NO:16)

**[0042]** Alternatively, at the 5′ end, the insertion is performed in such a way that the thyA promoter (Ross, 1990 a) is not included:

5′ tctgagaggttattttgggaaatacta**ttgaac**catatcgaggtgtgtgg**tataat**gaagggaattaaaaaagata**ggaaa**aat ttc**atg**...thyA wild-type (SEQ ID NO:29)

gattaagtcatcttacctctt...P1-T7-usp45-hIL10 (SEQ ID NO:29)

5′ tctgagaggttattttgggaaatactagattaagtcatcttacctctt...thyA⁻,P1-T7-usp45-hIL10 (SEQ ID NO:14)

**[0043]** At the 3′ end, an ACTAGT SpeI restriction site was engineered immediately adjacent to the TAA stop codon of the usp45-hIL-10 sequence. This was ligated in a TCTAGA *Xba*I restriction site, which was engineered immediately following the thyA stop codon

aaaatccgtaac**taactagt** 3′...usp45-hIL10 (SEQ ID NO:30)

gatttagcaatt**taa**attaaattaatctataagtt 3′...thyA-wild-type (SEQ ID NO:31)

**tctaga**attaatctataagttactga 3′...engineered thyA target (SEQ ID NO:32)

aaaatccgtaac**taactaga**attaatctataagttactga 3′...thyA⁻,usp45-hIL10 (SEQ ID NO:18)

**[0044]** These constructs are depicted in FIG. 2. The sequences of pOThy11, pOThy12 pOThy15 and pOThy16 are given by SEQ ID NOs: 23, 24, 25, and 26 respectively. The resulting strains are *thyA* deficient, a mutant not yet described for *L. lactis*. It is strictly dependent upon the addition of thymine or thymidine for growth.

**[0045]** The map of the deletion, as well as the PCR analysis of all the isolates/mutants of the present invention, is shown in FIGS. 3A-4B. The presence of the thymidylate synthase and the Interleukin 10 (IL-10) gene in the wild-type strain and in the independent isolates/mutant

was analyzed by Southern analysis as shown in FIGS. 5A-6B. The region around the inserted hIL-10 gene was isolated by PCR and the DNA sequence was verified. The structure is identical to the predicted sequence.

**[0046]** Human Interleukin-10 (hIL-10) production in the mutants was checked by western blot analysis and compared with the parental strain, transformed with pTREX1 as negative control, and the parental strain, transformed with the IL10-producing plasmid pT1HIL10apxa as a positive control (FIG. 7A). The concentration in the culture supernatant was quantified using ELISA. As shown in FIG. 7B, both isolates of the mutant produce a comparable, significant amount of hIL-10, be it far less than the strain, transformed with the nonintegrative plasmid pT1HIL10apxa. FIGS. 8A and 8B further demonstrate that all mutants produce a significant amount of hIL-10.

**[0047]** FIG. 9 shows the production of hIL-10 by the *L. lactis* strains LL108 carrying pOThy11, pOThy12, or pOThy16. Quantification (by ELISA) of hIL-10 present in the culture supernatant of the indicated strains is shown. The N-terminal protein sequence of the recombinant hIL-10 was determined by Edman degradation and was shown to be identical to the structure as predicted for the mature, recombinant hIL-10. The protein showed full biological activity. LL108 is an *L. lactis* strain carrying a genomic integration of the repA gene, required for replication of pORI19 derived plasmids such as pOThy11, pOThy12, pOThy15 or pOThy16. This strain was kindly donated by Dr. Jan Kok, University of Groningen, The Netherlands. The plasmids pOThy11, pOThy12, pOThy15 and pOThy16 carry the synthetic human IL-10 gene in different promoter configurations (see FIG. 2), flanked by approximately 1kB of genomic DNA derived from the thyA locus, upstream and downstream from thyA. These plasmids were used for the construction of the genomic integration as described.

**[0048]** The effect of the thymidylate synthase deletion on the growth in thymidine less and thymidine-supplemented media was tested; the results are summarized in FIGS. 10 and 11. An absence of thymidine in the medium strongly limits the growth of the mutant and even results in a decrease of colony-forming units after four hours of cultivation. The addition of thymidine to the medium results in an identical growth curve and amount of colony-forming units, compared to the wild-type strain, indicating that the mutant does not affect the growth or viability in thymidine-supplemented medium. FIG. 11 clearly demonstrates that Thy12 viability is severely impaired in the absence of thymidine.

**[0049]** FIG. 12 finally shows that *L. lactis* Thy12 passes through the intestine of the mice at the same speed as MG1363. Loss of viability does not appear to differ between Thy12 and MG1363. Thy12 appears fully dependent on thymidine for growth, indicating that no Thy12 bacteria had taken up a foreign thyA gene.

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